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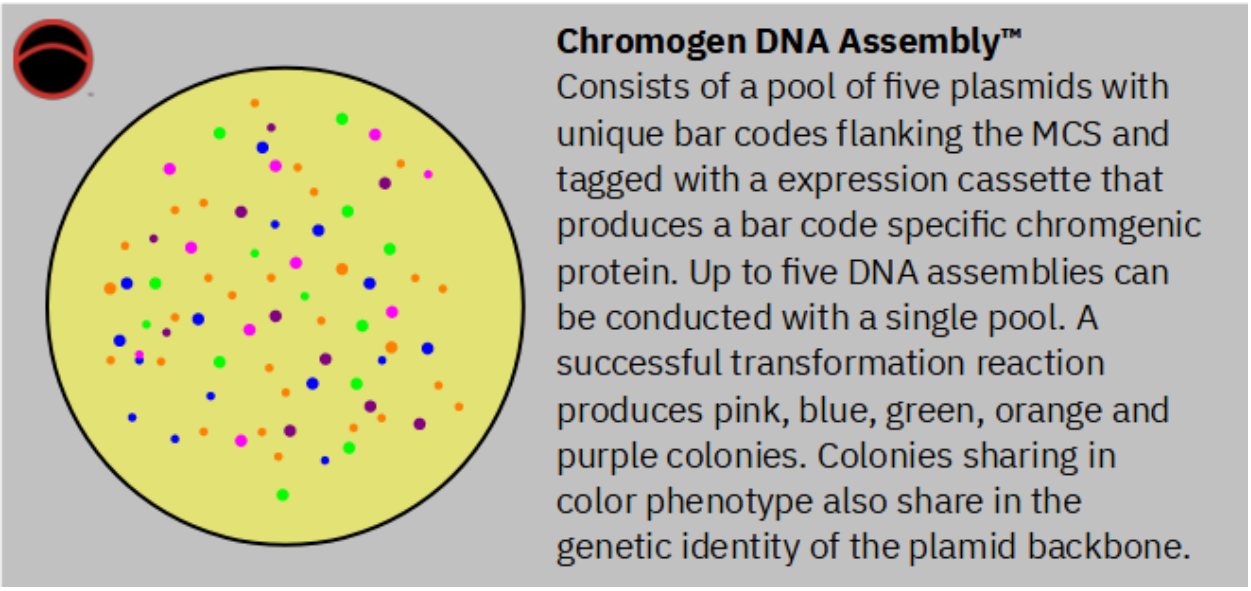
Radegen Biotechnology™


Chromogen DNA Assembly™
Consists of a pool of five plasmids with unique bar codes flanking the MCS and tagged with a expression cassette that produces a bar code specific chromgenic protein. Up to five DNA assemblies can be conducted with a single pool. A successful transformation reaction produces pink, blue, green, orange and purple colonies. Colonies sharing in color phenotype also share in the genetic identity of the plamid backbone.

10/10/2023

Chromogen DNA Assembly™

De novo assembly of multiple plasmids from oligonucleotide donor DNA.

Abstract:

De novo assembly of new genetic constructs using oligonucleotides has historically been conducted with a method called Polymerase Chain Assembly (PCA), a process that is time-consuming, expensive, and with sequence limitations. New methods for building new constructs have been proposed but have not been implemented on a wide scale in industry. Employment in industry requires new reagents and the establishment of modular DNA assembly paradigms in molecular biology. At the core of this paradigm is the concept of modularity of a DNA assembly reaction based on manufacturability instead of by functional categorization of genetic elements for making a new genetic regulatory element or protein coding sequence. Focusing attention on the ability of an end user to define every nucleotide in a strand of DNA expands possibilities not only in the synthesis of a never-before-seen peptide but also for new cloning strategies. Chromogenic DNA Assembly consists of a suit of selection plasmids that code for five distinct chromogenic proteins coded from an expression cassette flanking an MCS that is barcoded for targeting a DNA assembly to a distinct chromogen coding plasmid. This facilitates colony screening in multi-assembly

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transformation reactions by reducing the number of unnecessary duplicates that arise from randomly picking colonies and reduces cost of downstream steps by minimizing sequencing runs and consumable use. We predict that this system will have a positive effect on research budgets and the general well-being of laboratory workers by introducing fun visual stimuli, reduced workloads, greater direction and confidence when screening colonies, and new "water cooler topics" for friendly banter.

Introduction:

Chromogen DNA Assembly™ is Radegen Biotechnology's proprietary DNA assembly platform for the *de novo* synthesis of multiple DNA constructs from oligonucleotides in a one-pot reaction. This platform employs Radegen Biotechnology's proprietary DNA assembly reagent termed **Assemblyase™**, a RecA+ cell-free lysate for the *in vitro* assembly of DNA by **homology-directed repair (HDR)**. The platform incorporates a system of DNA assembly barcoded plasmids that code for **chromogenic proteins** that function as selection markers color coding the identity of the plasmid backbone harbored by a transformed *Escherichia coli* colony. The system consists of a pool of five plasmid backbones each coding for a green, blue, pink, orange or purple-colored chromogen that produces pigmented cells in a liquid medium or on an agar surface. This feature allows for the correct selection of a distinct plasmid backbone in a one-pot reaction assembling multiple DNA plasmids from a transformation reaction by selecting distinctly colored colonies. A unique set of homology arm barcodes is identified by a gene expressing one of the five colored chromogens and thus the genetic identity of the plasmid is known based on the colony color displayed from a plasmid pool set based on the homology arms assigned to a specific DNA cloning module. The plasmid system has a multiple cloning site (MCS) that modularizes the cloned DNA with either a NotI, SapI, BsaI or BsmBI restriction site for the downstream assembly of a larger genetic construct. A distinct oligo assembly is targeted to a specific destination plasmid by a set of homology arm barcodes adjacent to the MCS. **This system is designed for assembling DNA constructs up to 750 bp that can be used as qPCR positive controls, as a template for *in vitro* RNA synthesis, or as a Synthetic DNA Module™ for synthesizing a larger genetic construct by molecular cloning.**

Background:

Polymerase chain assembly (PCA) is the mainstream commercial method for assembling synthetic DNA constructs. This method has **limitations that make it difficult for a typical end user to adopt which include technical difficulty, time to a final construct, sequence design considerations, and cost**. The enzymatic nature of PCA forces end users to screen DNA sequences using bioinformatic tools that scan for features like GC content, repeat elements, and secondary structures to predict if a synthesis reaction is theoretically viable. PCA builds a dsDNA construct *in vitro* by multiple rounds of PCR. The final product from this process is not clonally pure and has an error rate similar to that produced by Taq polymerase (1 error per 5,000 bp), a condition that requires the use of a high-fidelity polymerase and results from **error accumulation after several hundred cycles of PCR**. An alternative method to assembling oligonucleotides by repetitive iterations of PCR is to assemble the constructs in a one-pot molecular cloning reaction in Radegen Biotechnology's Assemblyase™, a state-of-the-art RecA+ cell-free lysate for DNA assembly.

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Building dsDNA from ssDNA oligos by molecular cloning:

Assemblyase™ is Radegen Biotechnology's flagship and proprietary DNA assembly reagent specifically designed to assemble ssDNA oligonucleotides into dsDNA and clone into a plasmid. This reagent is composed of a cell-free lysate made from *Pseudomonas putida* EM42, a RecA+ "microbial cell-factory" strain with homologous recombination functionality comparable to that observed with *Myxococcus xanthus* and ***Saccharomyces cerevisiae***. The reagent is prepared like **SLiCE**, but is more powerful due to the engineered minimized genome focusing metabolic activity towards producing HDR and protein synthesis machinery. Assemblyase™ is capable of cloning dsDNA by seamless homology cloning into any plasmid and can produce up to 1 microgram of plasmid DNA from a single copy within 4 hours.

The mechanism for assembling DNA into a dsDNA construct employs the **DNA repair and replication machinery** found in the highly engineered EM42 strain and when harnessed into a reagent makes for a highly powerful high-fidelity process termed cloning by HDR. The **assembly of oligonucleotides by HDR cloning into a plasmid has been shown to be successful** in a *in vivo S. cerevisiae* system with specific limitations. The size of a final construct along with the size of the oligonucleotides used in assembly reactions were both found to be critical components. The size of an oligonucleotide is important because **longer oligonucleotides make for synthesis reactions with a lower percentage of full-length product**. Shorter oligos have a higher percentage of full-length products, facilitating the assembly. Sequence fidelity is not necessarily an issue because failed coupling reactions in a synthesis reaction are capped and removed from participating in subsequent coupling events producing a prep of truncations at each position along the molecule. The length of a DNA construct assembled from oligonucleotides has an inverse effect on the fidelity and reaction success rate due to misassembled products. The longer the DNA, the lower the percentage of correctly assembled products. Another important consideration in design is the length of the homology arms bridging overlapping sense and antisense oligonucleotides. Longer homology arms bridging oligonucleotides have been found to increase the success of a reaction and the fidelity of the products.

An industrial workflow for rational DNA assembly:

Chromogen DNA Assembly™ is an oligonucleotide ssDNA assembly platform rationally designed using the published collective body of literature for generating dsDNA between 25-750 bp in a chromogenic selection DNA assembly plasmid. The system is limited to the assembly of DNA constructs up to 750 bp for various reasons. A 750 bp upper size limitation facilitates high throughput DNA assembly in many ways: 1) increased efficiencies as measured by successfully synthesized construct over erroneous/failed assemblies, 2) facilitation of sanger sequencing with M13 sequencing primers, and 3) systemization of *in silico* design and benchwork.

The system assembles from one to eight oligonucleotides up to 130 nt in length with 30 nt hybridizing homology arms into a Chromogen DNA Assembly™ plasmid. The 5' and 3' oligonucleotides contain ~50 nt barcodes that target a construct assembly to a specific plasmid backbone. The system is specifically designed to work with oligo pools that contain donor DNA for multiple recombineered plasmids and where each construct is assembled into a distinct DNA assembly plasmid using special bar codes. Distinct plasmids are selected for when screening a transformation reaction by positive selection of colonies expressing a chromogenic protein. The Chromogen DNA Assembly™ plasmid suite contains a core set of five plasmids each expressing either a blue, green, purple, orange or pink

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chromogenic protein and each plasmid backbone is identified by a unique barcode that flanks the modularization module which is directly adjacent to the multiple cloning site where the user-defined DNA is to be assembled. The system is designed to be used with commercially available oligo pools from providers like Integrated DNA Technologies (Coralville, Iowa), Twist (San Francisco, California), and GenScript (Piscataway, New Jersey) and a pool consisting of the five Chromogen DNA Assembly™ plasmids. A transformation reaction results in a plate of *E. coli* colonies each harboring a plasmid either expressing a blue, green, purple, orange or pink chromogenic protein. These markers act as a positive selection marker for the presence of a recombineered plasmid and indicate the genetic distinctiveness of a colony on a plate transformed from a multi-assembly reaction.

Chromogen DNA Assembly™ system consists of 76 uniquely barcoded sets of pools containing 5 unique plasmids with unique selection markers and is specifically designed for **Integrated DNA Technologies' 50 pmol oPools** containing between 2-384 oligos or oligo pools with similar product features. Oligo length restrictions up to 130 nt is an optional rule but is highly recommended because the DNA preparation contains over 40% full-length product and synthesis time for a pool is right under 24 hours. This next-generation DNA assembly system is rationally designed using classical microbial molecular genetics concepts to facilitate high throughput synthetic *de novo* genetic construct assembly.

Applications:

- Synthetic DNA Module™ Synthesis
- qPCR positive control synthesis
- Sanger sequenced archive quality data storage at $40,000 per GB of storage space.

Discussion and Future Perspective.

Chromogen DNA Assembly™ is a DNA assembly and molecular cloning plasmid system designed for use with Assemblyase™. The system constitutes the formation of novel utility by bringing together multiple technologies like classical molecular genetics and synthetic biology to build a molecular workflow that deliberately reduces time, reduces consumable use, and introduces positive controls for manufacturing and end-user quality assurance. The end user can now pick five distinct colonies from a multi-assembly transformation reaction with confidence knowing that they are genetically distinct and one can re-pick a specific genotype if the original miniprep resulted in a plasmid with a mutation for resequencing with Sanger. This system also has the added advantage of adding aesthetic appeal and novelty to the cloning process, a feature that is predicted to **improve laboratory technician morale by increasing happiness stimulated by visual appeal and by a facilitated workload** when picking colonies.

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